

Resveratrol suppresses thyroid hormone-induced osteocalcin synthesis in osteoblasts

KAZUHIKO FUJITA^{1,2}, HARUHIKO TOKUDA^{2,3}, SHINGO KAINUMA^{1,2},
GEN KUROYANAGI^{1,2}, NAOHIRO YAMAMOTO^{1,2}, RIE MATSUSHIMA-NISHIWAKI²,
ATSUSHI HARADA⁴, OSAMU KOZAWA² and TAKANOBU OTSUKA¹

¹Department of Orthopedic Surgery, Nagoya City University Graduate School of Medical Sciences, Nagoya, Aichi 467-8601; ²Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194; Departments of ³Clinical Laboratory and ⁴Orthopedic Surgery, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8511, Japan

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Abstract. Resveratrol, a polyphenolic compound that is present in grape skins, berries and red wine, may be beneficial for human health through its anti-inflammatory and anti-oxidant effects. It has been previously demonstrated that resveratrol exerts its biological effects primarily via sirtuin 1 (SIRT1) activation. We previously reported that triiodothyronine (T₃) induces osteocalcin synthesis in osteoblast-like MC3T3-E1 cells, and that p38 mitogen-activated protein (MAP) kinase mediates the T₃-stimulated synthesis of osteocalcin. The present study investigated the effect of resveratrol on T₃-induced osteocalcin synthesis and its underlying mechanism in MC3T3-E1 cells. Cultured cells were stimulated with T₃, and osteocalcin release from MC3T3-E1 cells was measured by ELISA and phosphorylation of p38 MAP kinase was analyzed by western blotting. Resveratrol significantly suppressed the release of osteocalcin stimulated by T₃, and SIRT1720, a SIRT1 activator, significantly reduced T₃-induced osteocalcin release. The expression level of osteocalcin mRNA stimulated by T₃ was significantly attenuated by resveratrol and T₃-induced transactivation activity of the thyroid hormone-responsive element was significantly diminished by resveratrol. However, only limited effects of resveratrol on the T₃-induced phosphorylation of p38 MAP kinase were observed. The results of the present study demonstrated that resveratrol suppresses T₃-stimulated osteocalcin synthesis at a point upstream of transcription in osteoblasts, and that the inhibitory effect of resveratrol is mediated, at least partially, through SIRT1 activation. These results indicate that there may be a novel role for the polyphenol in the modulation of bone metabolism.

Introduction

Resveratrol is a natural polyphenolic compound that is present in grapes, berries and red wine. Accumulating evidence indicates that resveratrol may provide various health benefits, including protective properties against cardiovascular disease and cancer (1,2). The majority of the anti-apoptotic or anti-inflammatory effects of resveratrol are associated with the activation of sirtuin 1 (SIRT1), which is known as a nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylase that activates genes associated with longevity and survival (3,4). The French population reportedly tends to consume high levels of saturated fatty acids in their meals however, their circulating levels remain relatively low and they have low mortality rates associated with coronary heart disease, when compared with other countries that have a similar consumption of saturated fats. This may be due to their frequent consumption of red wine containing abundant resveratrol (5).

As for the effects of resveratrol on bone cells, resveratrol is reported to stimulate the differentiation of osteoblasts (6,7), which conduct bone formation and also bone resorption through the expression of receptor activator of nuclear factor-κB (RANK)-ligand (RANKL), which binds to RANK expressed on osteoclasts (8). Previous reports from this lab have demonstrated that, in osteoblast-like MC3T3-E1 cells, resveratrol suppresses the synthesis of osteoprotegerin (OPG), a decoy receptor of RANKL (8), induced by prostaglandin F_{2α} (PGF_{2α}), PGD₂, PGE₁, PGE₂ or basic fibroblast growth factor (FGF-2) (9-13). However, resveratrol increases bone morphogenetic protein-4 (BMP-4)-induced OPG synthesis (14). In addition, synthesis of vascular endothelial growth factor (VEGF) induced by BMP-4 or transforming growth factor-β (TGF-β) is inhibited by resveratrol in MC3T3-E1 cells (15,16). These findings indicate that resveratrol may orchestrate stimuli from numerous bone remodeling agents, resulting in the modulation of bone metabolism. However, the exact mechanism underlying the effects of resveratrol on osteoblasts remains to be elucidated.

Osteocalcin, which is synthesized in osteoblasts, is the most abundant non-collagenous protein and is also a marker

Correspondence to: Dr Haruhiko Tokuda, Department of Clinical Laboratory, National Center for Geriatrics and Gerontology, 7-430 Morioka-cho, Obu, Aichi 474-8511, Japan
E-mail: tokuda@ncgg.go.jp

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of mature osteoblast phenotype (17). Osteocalcin undergoes post-translational modification whereby glutamic acid residues are carboxylated to form γ -carboxylglutamic acid (Gla) residues by vitamin K-dependent γ -carboxylase (17). It has been reported that osteocalcin-deficient mice present higher bone mass and bone strength, indicating that osteocalcin is a determining factor in bone formation (18). In addition, it was previously proposed that uncarboxylated osteocalcin released from osteoblasts functions as a hormone, which regulates energy metabolism by stimulation of insulin secretion from pancreatic β -cells and by upregulation of insulin sensitivity through adiponectin production by adipocytes (19). Thus, the evidence indicates that bone may also have a crucial role as an endocrine organ, regulating energy metabolism through the production of osteocalcin by osteoblasts (20).

In addition to a role in the modulation of whole-body metabolism, thyroid hormone is an important modulator of skeletal function. Excess levels of thyroid hormone, which is termed hyperthyroidism, accelerates metabolic turnover rate and increases the ratio of bone resorption to bone formation, which may lead to osteoporosis (21,22). It was previously reported that bone mineral density is significantly decreased and the risk of fracture increases in untreated patients with hyperthyroidism (23). The thyroid hormone receptor is a member of the steroid hormone receptor superfamily (24). It is established that thyroid hormone, like other steroid hormones, binds to its specific intracellular receptors and the complex subsequently induces the expression of the gene network (24). In our previous study (25), triiodothyronine (T_3) stimulated osteocalcin synthesis in osteoblast-like MC3T3-E1 cells and p38 mitogen-activated protein (MAP) kinase positively regulated the synthesis. The present study investigated the effect of resveratrol on T_3 -stimulated osteocalcin synthesis in MC3T3-E1 cells. The results of the present study indicate that resveratrol may suppress T_3 -stimulated osteocalcin synthesis at a point upstream of transcription in osteoblast-like MC3T3-E1 cells, and that the inhibition by resveratrol is mediated, at least partially, by SIRT1 activation.

Materials and methods

Materials. T_3 was obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Resveratrol and SRT1720 were obtained from EMD Millipore (Billerica, MA, USA). The mouse osteocalcin ELISA kit (cat. no. BT-470) was obtained from Alfa Aesar; Thermo Fisher Scientific, Inc. (Lancashire, UK). Phospho-specific p38 MAP kinase (cat. no. #4511) and p38 MAP kinase (cat. no. #9212) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (cat. no. sc-25778) antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). An enhanced chemiluminescence (ECL) western blotting detection system was obtained from GE Healthcare Life Sciences (Chalfont, UK). Other materials and chemicals were obtained from commercial sources. T_3 was dissolved in 0.1 M NaOH. Resveratrol and SRT1720 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the assay for osteocalcin,

reverse transcription-quantitative polymerase chain reaction (RT-qPCR), luciferase reporter assay or western blot analysis.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells that were derived from newborn mouse calvaria (26) were maintained as previously described (27). Briefly, the cells were cultured in α -minimum essential medium (α -MEM) containing 10% fetal bovine serum (FBS; cat. no. 12483-020; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere of 5% CO_2 /95% air. The cells were seeded into 35 mm diameter dishes (5×10^4 cells/dish) or 90 mm diameter dishes (2×10^5 cells/dish) in α -MEM containing 10% FBS. After 5 days, the medium was exchanged for α -MEM containing 0.3% FBS; the cells were used for experiments 48 h following this exchange.

Assay for osteocalcin. Cultured cells were pretreated with various doses of resveratrol (0, 10, 50 and 70 μ M), or SRT1720 at 37°C for 60 min, and subsequently stimulated by 10 nM of T_3 or vehicle in 1 ml of α -MEM containing 0.3% FBS at 37°C for 0, 48 or 96 h. In the experiment investigating the effect of various resveratrol doses on osteocalcin release, cells were stimulated by T_3 or vehicle for 96 h, whereas cells pretreated with SRT170 were stimulated by T_3 or vehicle for 0, 48 or 96 h. The conditioned medium was collected at the end of incubation and the osteocalcin concentration in the medium was measured using the mouse osteocalcin ELISA kit according to the manufacturer's protocol.

RT-qPCR. Cultured cells were pretreated with 50 μ M of resveratrol or vehicle at 37°C for 60 min, and subsequently stimulated by 10 nM of T_3 or vehicle in 1 ml of α -MEM containing 0.3% FBS for 48 h. Total RNA was isolated and transcribed into cDNA using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and Omniscript[®] RT kit (Qiagen, Inc., Valencia, CA, USA), respectively. qPCR was performed using a LightCycler[®] Fast Start DNA Master SYBR Green I kit in capillaries (Roche Diagnostics, Basel, Switzerland). The forward and reverse primers for mouse osteocalcin mRNA were synthesized based on the reports by Zhang *et al.* (28) and were obtained from Greiner Bio-One Co., Ltd. (Tokyo, Japan). These primer sequences (listed 5'-3') were as follows: forward, TTCTGCTCACTCTGCTGACC and reverse, TTTGTAGGC GGTCTTCAAGC. The forward and reverse primers for mouse GAPDH mRNA were synthesized based on the report by Simpson *et al.* (29) and were obtained from Sigma-Aldrich; Merck KGaA. These primer sequences (listed 5'-3') were as follows: forward, AACGACCCCTTCATTGAC and reverse, TCCACGACATACTCAGCAC. The reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles at 60°C for 5 sec and 72°C for 7 sec. The amplified products were determined by melting curve analysis (30), according to the system protocol. The data were analyzed using the second derivative maximum method and LightCycler3 Data Analysis software (version 3.5.28; Roche Diagnostics). The osteocalcin mRNA levels were normalized to those of GAPDH mRNA using the pfaffl method (31).

Luciferase reporter assay. A reporter plasmid, *pDR4* (thyroid hormone-responsive element)-*Luc* was purchased

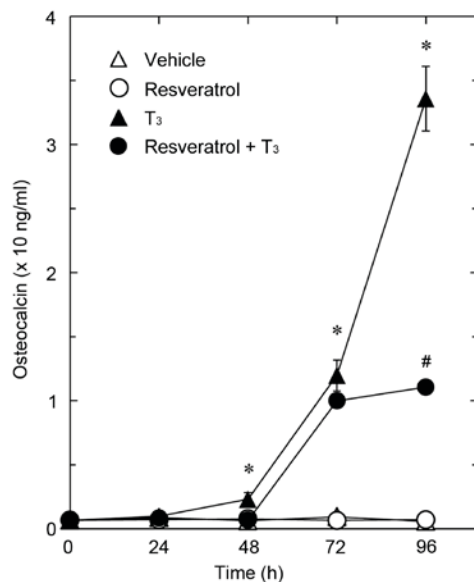


Figure 1. Effect of resveratrol on T₃-stimulated osteocalcin release in MC3T3-E1 cells. Cultured cells were pretreated with 50 μ M of resveratrol (●,○) or vehicle (▲,△) for 60 min, and subsequently stimulated with 10 nM of T₃ (●,▲) or vehicle (○,△) for 0, 24, 48, 72 or 96 h. Osteocalcin concentrations in the conditioned medium were determined by ELISA. Results are presented as the mean \pm standard error of the mean of triplicate results from three independent cell preparations. *P<0.05 vs. vehicle only and #P<0.05 vs. T₃ only. T₃, triiodothyronine.

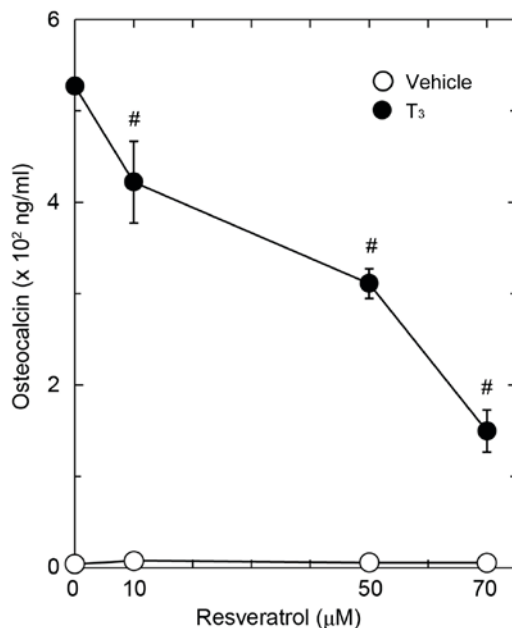


Figure 2. Effect of various doses of resveratrol on T₃-stimulated osteocalcin release in MC3T3-E1 cells. Cultured cells were pretreated with various doses of resveratrol for 60 min and subsequently stimulated with 10 nM of T₃ (●) or vehicle (○) for 96 h. Osteocalcin concentrations in the conditioned medium were determined by ELISA. Results are presented as the mean \pm standard error of the mean of triplicate results from three independent cell preparations. #P<0.05 vs. T₃ only. T₃, triiodothyronine.

from Stratagene (Agilent Technologies, Inc., Santa Clara, CA, USA). The cultured cells were pretreated with 50 μ M resveratrol or vehicle at 37°C for 6 h after the transfection with the *pDR4-Luc* reporter plasmid (1 μ g/dish) using UniFactor

transfection reagent at 37°C for 6 h (B-Bridge International, Inc., Santa Clara, CA, USA). The cells were cotransfected with pRL-CMV (*Renilla* luciferase; 0.1 μ g/dish; Promega Corporation, Madison, WI, USA) as an internal standard to normalize transfection efficiency. Following pretreatment, cells were stimulated by 10 nM T₃ or vehicle at 37°C for 48 h. Luciferase activity of the cell lysates was measured using a Dual-Luciferase® Reporter Assay system (Promega Corporation) according to the manufacturer's protocol.

Western blot analysis. The cultured cells were pretreated with various doses of resveratrol (0, 10, 30 and 50 μ M) at 37°C for 60 min and subsequently stimulated by 10 nM of T₃ in a-MEM containing 0.3% FBS at 37°C for 120 min. Cells were washed twice with PBS and lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% SDS, 50 mM dithiothreitol and 10% glycerol. SDS-PAGE was performed as described by Laemmli (32) on 10% polyacrylamide gels. Protein quantification was performed using a Pierce Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Protein (10 μ g/lane) was fractionated and transferred onto an Immun-Blot® PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% fat-free dry milk in TBS-Tween-20 (TBS-T; 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) at 37°C for 1 h prior to incubation with primary antibodies. Western blot analysis was performed as described previously (33) using phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies or GAPDH as primary antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG (KPL, Inc., Gaithersburg, MD, USA) used as secondary antibodies. The primary and secondary antibodies were diluted at 1:1,000 with 5% fat-free dry milk in TBS-T. The peroxidase activity on the PVDF sheet was visualized on X-ray film by means of the ECL western blotting detection system (GE Healthcare Life Sciences) according to the manufacturer's protocol.

Statistical analysis. Results were analyzed by one-way analysis of variance followed by the Bonferroni method for multiple comparisons between pairs, using Microsoft Office Excel 2013 for Windows (Microsoft Corporation, Redmond, WA, USA) and P<0.05 was considered to indicate a statistically significant difference. Results are presented as the mean \pm standard error of the mean of triplicate results from three independent cell preparations.

Results

Effect of resveratrol on the T₃-stimulated osteocalcin release in MC3T3-E1 cells. The present study first investigated the effect of resveratrol on T₃-stimulated osteocalcin release in osteoblast-like MC3T3-E1 cells. As previously described (25), T₃ stimulated secretion of osteocalcin after 48 h. Resveratrol, which had a limited effect on the release of osteocalcin alone, significantly reduced T₃-stimulated release of osteocalcin compared with cells treated with T₃ only (Fig. 1). The suppressive effect of resveratrol on the T₃-stimulated osteocalcin release was dose-dependent in the range between 10 and 70 μ M (Fig. 2). The maximum effect of resveratrol was

observed at 70 mM, which caused a ~70% decrease in the T_3 -effect.

Effect of SRT1720 on the T_3 -stimulated osteocalcin release in MC3T3-E1 cells. It has been previously reported that resveratrol exerts its biological effects through the activation of SIRT1 (1,2). Thus, the present study investigated the effect of SRT1720, which is a specific and potent synthetic activator of SIRT1 (34), on T_3 -stimulated osteocalcin release in osteoblast-like MC3T3-E1 cells. Similarly to resveratrol, SRT1720 significantly reduced T_3 -stimulated osteocalcin synthesis compared with cells treated with T_3 only (Fig. 3).

Effect of resveratrol on T_3 -induced expression of osteocalcin mRNA in MC3T3-E1 cells. In order to investigate whether the inhibitory effect of resveratrol on the T_3 -stimulated osteocalcin release is exerted through transcriptional events or not, the present study examined the effect of resveratrol on T_3 -induced osteocalcin mRNA expression by RT-qPCR. Resveratrol, which had no effect on basal levels of osteocalcin mRNA when applied alone, significantly reduced the expression level of osteocalcin mRNA induced by T_3 compared with cells treated with T_3 only (Fig. 4).

Effect of resveratrol on T_3 -induced transactivation activity of thyroid hormone-responsive element in MC3T3-E1 cells. In addition, the effect of resveratrol on T_3 -stimulated transactivation activity of thyroid hormone-responsive element in osteoblast-like MC3T3-E1 cells was investigated using a luciferase reporter assay. Pretreatment with resveratrol alone had a limited effect on the luciferase activity of thyroid hormone-responsive element compared with untreated cells (Fig. 5). However, resveratrol significantly inhibited the activity induced by T_3 compared with cells treated with T_3 only (Fig. 5).

Effect of resveratrol on the T_3 -induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. In our previous study, T_3 -stimulated osteocalcin synthesis was demonstrated to be positively regulated through the p38 MAP kinase pathway in osteoblast-like MC3T3-E1 cells (25). In order to clarify whether the activation of p38 MAP kinase is implicated in the resveratrol-effect on T_3 -induced osteocalcin synthesis in these cells, the present study investigated the effect of resveratrol on the T_3 -induced phosphorylation of p38 MAP kinase. However, the results demonstrated that resveratrol did not affect the T_3 -stimulated phosphorylation of p38 MAP kinase (Fig. 6).

Discussion

The present study demonstrated that resveratrol significantly decreased T_3 -stimulated osteocalcin release in osteoblast-like MC3T3-E1 cells. Additionally, it was observed that resveratrol significantly reduced osteocalcin mRNA levels upregulated by T_3 . Therefore, the inhibitory effect of resveratrol on the T_3 -induced osteocalcin synthesis may occur upstream of transcriptional levels in osteoblast-like MC3T3-E1 cells. A number of biological effects of resveratrol are reported to be dependent on SIRT1 activation (1,2). Therefore, in order to investigate whether the suppression of T_3 -stimulated osteocalcin synthesis

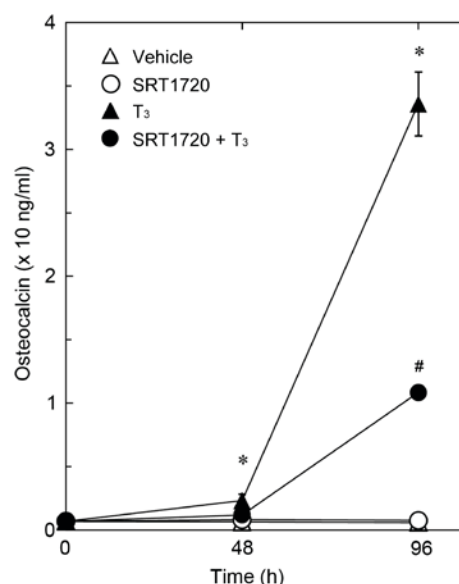


Figure 3. Effect of SRT1720 on T_3 -stimulated osteocalcin release in MC3T3-E1 cells. Cultured cells were pretreated with 10 μ M of SRT1720 (●,○) or vehicle (▲,△) for 60 min, and subsequently stimulated with 10 nM of T_3 (●,▲) or vehicle (○,△) for 0, 48 or 96 h. Osteocalcin concentrations in the conditioned medium were determined by ELISA. Results are presented as the mean \pm standard error of the mean of triplicate results from three independent cell preparations. * P <0.05 vs. vehicle only and # P <0.05 vs. T_3 only. T_3 , triiodothyronine.

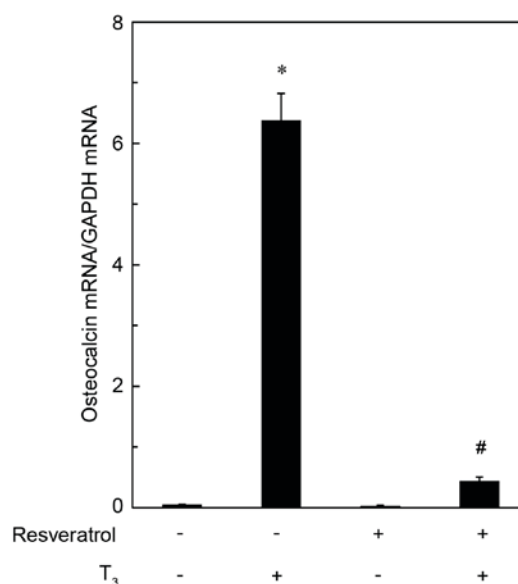


Figure 4. Effect of resveratrol on T_3 -induced expression levels of osteocalcin mRNA in MC3T3-E1 cells. Cultured cells were pretreated with 50 μ M of resveratrol or vehicle for 60 min, and subsequently stimulated with 10 nM of T_3 or vehicle for 48 h. The expression of osteocalcin mRNA and GAPDH mRNA were quantified by reverse transcription-quantitative polymerase chain reaction and osteocalcin mRNA levels were normalized to GAPDH mRNA levels. Results are presented as the mean \pm standard error of the mean of triplicate results from three independent cell preparations. * P <0.05 vs. control and # P <0.05 vs. T_3 only. T_3 , triiodothyronine.

by resveratrol is mediated through SIRT1 in MC3T3-E1 cells, the effect of SRT1720, a synthetic compound that activates SIRT1 with a potency 1,000-fold greater than resveratrol (34), on the release of osteocalcin was examined. The results

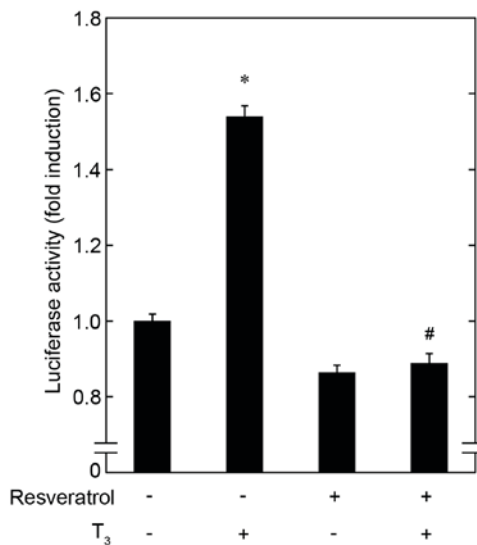


Figure 5. Effect of resveratrol on T_3 -induced transactivation activity of thyroid hormone-responsive element in MC3T3-E1 cells. Cultured cells were pretreated with 50 μ M resveratrol or vehicle at 6 h after transfection with the pDR4-Luc reporter plasmid (1 μ g/dish). Following the pretreatment for 60 min, cells were stimulated with 10 nM T_3 or vehicle for 48 h. A luciferase reporter assay was conducted. Values are presented as fold induction of luciferase activity compared with the control and as the mean \pm standard error of the mean of triplicate results from three independent cell preparations. * P <0.05 vs. vehicle only and # P <0.05 vs. T_3 only. T_3 , triiodothyronine.

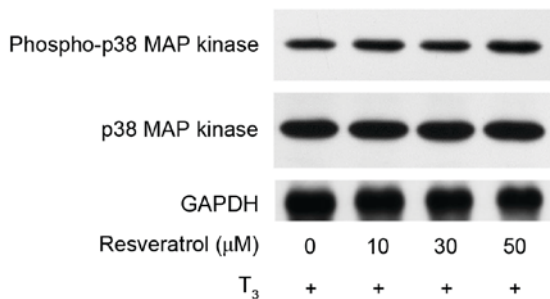


Figure 6. Effect of resveratrol on T_3 -induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. Cultured cells were pretreated with 0, 10, 30 or 50 μ M resveratrol for 60 min, and subsequently stimulated with 10 nM of T_3 for 120 min. Cell extracts were subjected to SDS-PAGE, with subsequent western blot analysis with antibodies against phospho-specific p38 MAP kinase, p38 MAP kinase, or GAPDH. T_3 , triiodothyronine; MAP kinase, mitogen activated protein kinase.

demonstrated that SRT1720 mimicked the suppressive effect of resveratrol on T_3 -induced release of osteocalcin. Based on the results, the inhibitory effect of resveratrol on osteocalcin synthesis induced by T_3 may be mediated, at least partially, by the activation of SIRT1 in osteoblast-like MC3T3-E1 cells.

It is established that the effects of thyroid hormone, a member of the nuclear receptor superfamily, are exerted via its binding to specific receptors in the nucleus, and that the receptor-hormone complex subsequently activates target gene expression (35). Therefore, the present study investigated the effect of resveratrol on T_3 -induced transactivation activity of thyroid hormone-responsive element by a luciferase reporter assay in MC3T3-E1 cells. Resveratrol significantly reduced the T_3 -induced transactivation activity of thyroid hormone-responsive element, in addition to the expression of

osteocalcin mRNA in MC3T3-E1 cells. The results indicate that the inhibitory effect of resveratrol on T_3 -induced osteocalcin synthesis may be exerted upstream of gene transcription in osteoblast-like MC3T3-E1 cells.

In the MAP kinase superfamily, it is established that p44/p42 MAP kinase, p38 MAP kinase and stress-activated protein kinase/c-Jun N-terminal kinase have pivotal roles in a variety of cellular functions, including proliferation, differentiation and survival (36). Our previous studies (25,37) reported that T_3 stimulates the activation of p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells, and that p38 MAP kinase, and not p44/p42 MAP kinase, functions as a positive regulator in osteocalcin synthesis stimulated by T_3 . However, the present study demonstrated that resveratrol did not affect the T_3 -induced phosphorylation of p38 MAP kinase in these cells. Based on the results, it seems unlikely that the effect of resveratrol on T_3 -stimulated osteocalcin synthesis is exerted at a point upstream of p38 MAP kinase in osteoblast-like MC3T3-E1 cells.

Osteocalcin is synthesized specifically in mature osteoblasts and stored in the bone matrix (17). The presence of the three Glu residues is critical for the structure and function of osteocalcin, which allows it to bind to hydroxyapatite with a high affinity in their fully carboxylated state, which subsequently regulates the maintenance of adequate bone mass (18). Therefore, the results of the present study, which demonstrate suppression of T_3 -stimulated osteocalcin synthesis by resveratrol, may indicate a novel role for the polyphenol in the modulation of bone metabolism. We have recently demonstrated that resveratrol modulates the synthesis of OPG stimulated by $\text{PGF}_{2\alpha}$, PGD_2 , PGE_1 , PGE_2 , FGF-2 or BMP-4, and regulates VEGF synthesis induced by BMP-4 or TGF- β in osteoblast-like MC3T3-E1 cells (9-16). Taking these findings into account, resveratrol may support the maintenance of skeletal conditions via orchestrating osteoblast functions elicited by various stimuli, including prostaglandins, cytokines and growth factors, which may explain the lower hip bone fracture risk observed in wine drinkers (6). In addition, osteocalcin has recently been recognized as a potent bone-derived hormone that regulates energy or lipid metabolism (20). Based on the results of the current study, as T_3 -stimulated osteocalcin synthesis was suppressed by resveratrol in osteoblast-like MC3T3-E1 cells, resveratrol may modulate whole body energy metabolism through the regulation of osteocalcin synthesis in osteoblasts. Further investigation is required to clarify the exact mechanism underlying the effects of resveratrol in osteoblasts. In conclusion, the results of the present study indicate that resveratrol suppresses T_3 -stimulated osteocalcin synthesis upstream of transcription in osteoblasts, and that the inhibitory effect of resveratrol is partially mediated by SIRT1.

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